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The results of the Phase I investigation have met, and in many areas surpassed, the initial expectations of our proposal. These results include: 1) development of a near market-ready biosensor carcinogen detection system for use as a general screening test; 2) detection of Ethidium Bromide in the 1 μg/ml (1 ppm) range with a yet-to-be optimized genetically cloned sensor; 3) lyophilization and very successful rehydration of cloned biosensors after 1.5 months of storage; 4) early progress in the development of a mercury sensor, which establishes a methodology for the development of a variety of specific biosensors; 5) development of several light detection systems that include two photodiode based systems, two battery-powered photomultiplier system, and two film techniques.

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Bioluminescence for Detection of Trace Compounds

Contract Number: N00014-89-C-0251

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INTRODUCTION

Development of a detection system that is highly sensitive, specific, and easy to use is of significant importance in a variety of applications in environmental evaluation chemical screening, production control, and research. BioPhotonics' Phase I research focused on creating such a sensor by coupling the inherent sensitivity of biological systems to carcinogens and heavy metals with the ability of certain bacteria to emit light. Using recombinant DNA techniques, BioPhotonics has successfully isolated and fused specific sensing genes to genes responsible for light emission, thus producing uniquely tailored biosensors. This technology, combined with the development of an economical and portable light detection unit, has produced a biologically based detection system that can sense and respond to low concentrations of the broad class of toxics that are carcinogens. More importantly, the principles and techniques developed during Phase I can be readily applied to the construction of biosensors for the detection of many other types of compounds, including toxic chemicals (organics and inorganics), heavy metals, and other bioactive molecules.

PHASE I TECHNICAL OBJECTIVES

Three primary goals were outlined in the Phase I proposal. The first objective was to demonstrate the feasibility of utilizing cloned *lux* genes from *Photobacterium leiognathi* (a naturally luminous bacterium) to generate a general screening test that responds to the presence of carcinogens by emitting visible light. The second objective was to design and fabricate a light detection system of sufficient sensitivity to detect low level light responses. The final objective was to initiate the construction of a chemical specific sensor; this task involved forming genetic fusions of *mer* (mercury resistant) and *lux* genes to create a precursor for the development of a mercury specific biosensor.

RESEARCH ACCOMPLISHMENTS

Detection of Carcinogens Using Cloned lux Genes in E. coli

A variety of *E. coli* strains were used to generate carcinogen sensitive biosensors (Table 1). These *E. coli* strains were transformed with a plasmid containing the five structural genes for bacterial luminescence (*lux* operon; Figure 1). A blue-green light is produced by the enzyme luciferase, which is translated from the *luxA* and *luxB* genes (Hastings and Nealson, 1977; Hastings et al., 1978; Baldwin et al., 1988). The plasmid pSD721, which contains cloned genes from *Photobacterium leiognathi* PL721 ligated into the plasmid pACYC184 (Figure 2), was selected for this research because of its known response to carcinogens (Delong et al. 1987). Throughout this investigation, host bacteria and clones were grown at 30°C under continuous agitation in Luria Broth (LB), which contains 10 g of Bacto-Tryptone, 5 g of Bacto-yeast extract, and 10 g of NaCl per liter of broth.

Table 1. Strains and plasmids used in the carcinogen study.

Strain or Plasmid	Genotype	Source
E. coli Strain		
DH5α	F^-, ϕ80/*lacZ*ΔM15, *endA*1, *recA*1, *hsdR*17(r_k^-, m_k^-), *supE*44, *thi*-1, *gyrA*, *relA*1, Δ(*lacZYA-argF*), U169, λ^-	Saffarini
LE392	F^-, *hsdR*514(r_k^-, m_k^-), *supE*44, *supEF*58, *lacY*1, *galK*2, *galT*22, *metB*1, *trpR*55, λ^-	Nealson
Plasmid		
pSD721	*luxC.D.A.B.G.E*; from *P. leiognathi*	Nealson

THE BACTERIAL BIOLUMINESCENCE (*lux*) REGULON IN Vibrio Fischeri

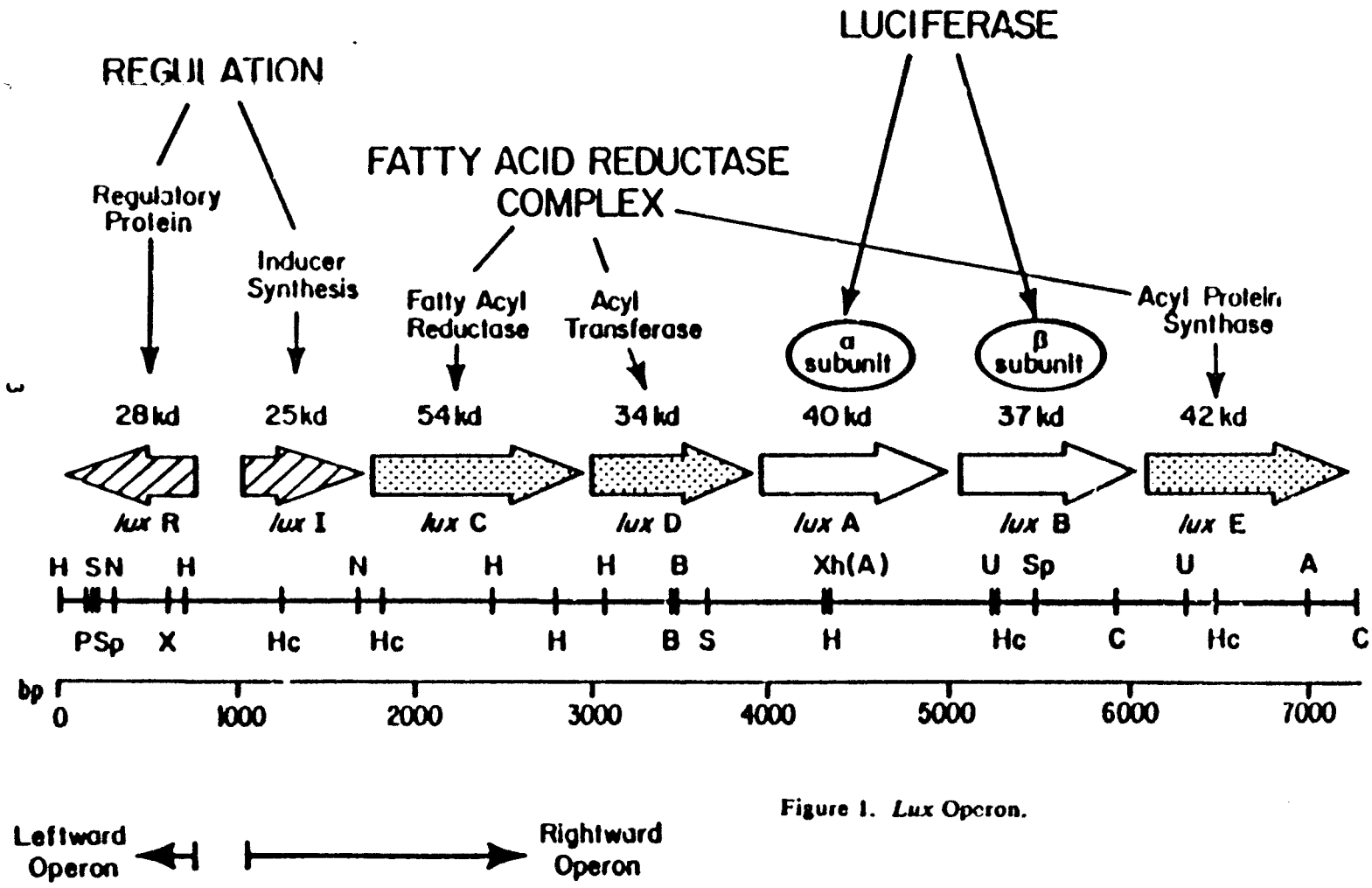

Figure 1. *Lux* Operon.

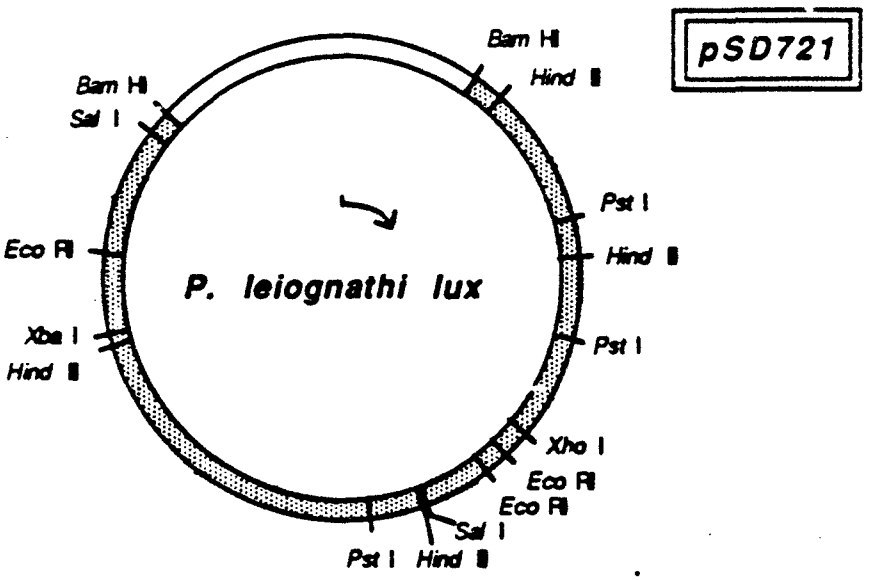

Figure 2. Map of the plasmid, pSD721, which contains inserted *lux* genes from *Photobacterium leiognathi*.

Carcinogen Biosensor Construction

The transformation of *E. coli* was preformed by electroporation (Calvin and Hanawalt, 1988). Cultures of *E. coli* were first diluted into 50 ml of fresh LB at 30°C and then grown overnight to an optical density of 0.5 measured at λ = 600 nm (OD_{600} = 0.5). Host *E. coli* cells were harvested by centrifugation and washed using a sucrose electroporation buffer (pH 7.4), containing 272 mM sucrose and 10 mM HEPES. The *E. coli* cells were suspended in 1 ml of the sucrose buffer along with 2 μg of the purified pSD721 and then held on ice for 10 minutes, prior to electroporation at 50 μF and 2,400 V. The electroporated cell suspensions were allowed to recover for 30 to 60 minutes on ice and then were diluted 5 fold in LB and allowed to grow for 1 hour at 30°C. Finally, the suspensions were diluted, plated onto LB plates containing 100 μg/ml Ampicillin (Amp), and allowed to grow overnight. The surviving clones are both Amp-resistant and luminescent, and thus are potential biosensors.

Assay for Bioluminescence and Luminescent Response to Carcinogens

Bioluminescence assay conditions

All luminescence measurements were performed using 1 ml suspensions of bacteria in 20 ml glass vials. Since oxygen is an essential substrate for the bacterial luciferase (Hastings and Nealson, 1977; Hastings et al., 1978) and is ideally present in excess, a large surface area to volume ratio is necessary. Luminescence was measured by placing the filled vials in a small light-tight chamber which houses a photomultiplier tube (EMI 9781A). The photomultiplier output was measured using a Pacific Photometrics (110) amplifier and an analog current meter. This system was calibrated using the light standard of Hastings and Webber (1963); luminescence is expressed in quanta per second (qs^{-1}). The optical density (OD_{600}), which is proportional to the cell number of the suspension, was measured in a LKB Ultraspec 4050 spectrophotometer, using λ = 600 nm.

The luminescent response to carcinogens

Ethidium Bromide (EtBr) was selected as the standard carcinogenic material for these investigations for several reasons: the properties of EtBr are representative of many intercalating carcinogens, EtBr is relatively safe to handle under laboratory conditions, and EtBr's affects on these types of clones have been studied previously.

The Luminescent Response to EtBr During Growth. The expression of pSD721 *lux* genes in a recombinant host is strongly dependent on the specific host into which the *lux* genes are transformed (Delong et al., 1987). Some clones are virtually dark, while others are nearly as luminous as the bacterium from which the *lux* genes are cloned (Figure 3). Luminescence can also be stimulated by the presence of carcinogens such as EtBr (Figure 4). A similar response to carcinogens has also been noted in a related natural marine luminous bacterium, *P. phosphoreum* (Ulitzur and Weiser, 1981; Ulitzur et al., 1981; Ulitzur, 1986). While the molecular and regulatory basis for this response is not well understood at this time, the response is reproducible (Ulitzur et al., 1981; Ulitzer, 1986; Steinberg et al., 1985; Steinberg et al., 1988).

Review of the literature convinced BioPhotonics that the pSD721 cloned *lux* system would be an excellent candidate for development of a biosensor sensitive to carcinogens. In this study, the plasmid pSD721 was transformed into several previously untested hosts to create clones that respond rapidly and strongly to the presence of EtBr, while maintaining a nearly nonluminous background response. Of the various clones constructed, three clones were chosen for further study: *E. coli* DH5α, *E. coli* LE392, and *E. coli* WB373. For all of the clones tested, the culture density doubled every 1.0 to 1.75 hours (Figure 5), and in each case, the doubling times were essentially the same with and without EtBr; hence, these clones can respond to a carcinogen without significant inhibition of their growth and metabolism.

One advantage of using luminescence as a biosensor reporter is that synthesis and expression of bacterial luciferase is exponentially, rather than proportionally related to cell

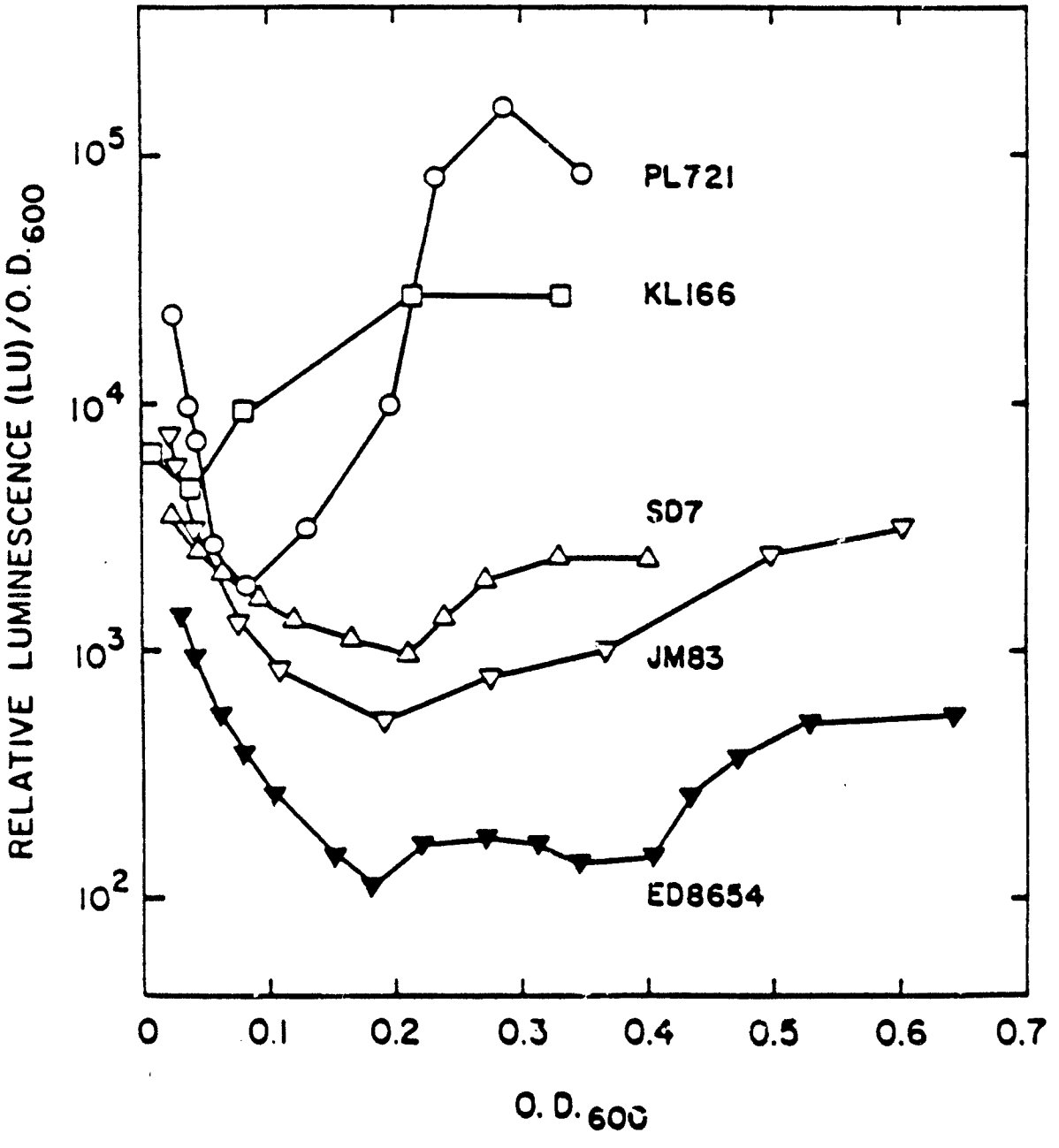

Figure 3. Effect of host strain on level of expression of pSD721 luminescense produced by *Photobacterium leiognathi* and by the cloned *lux* genes.

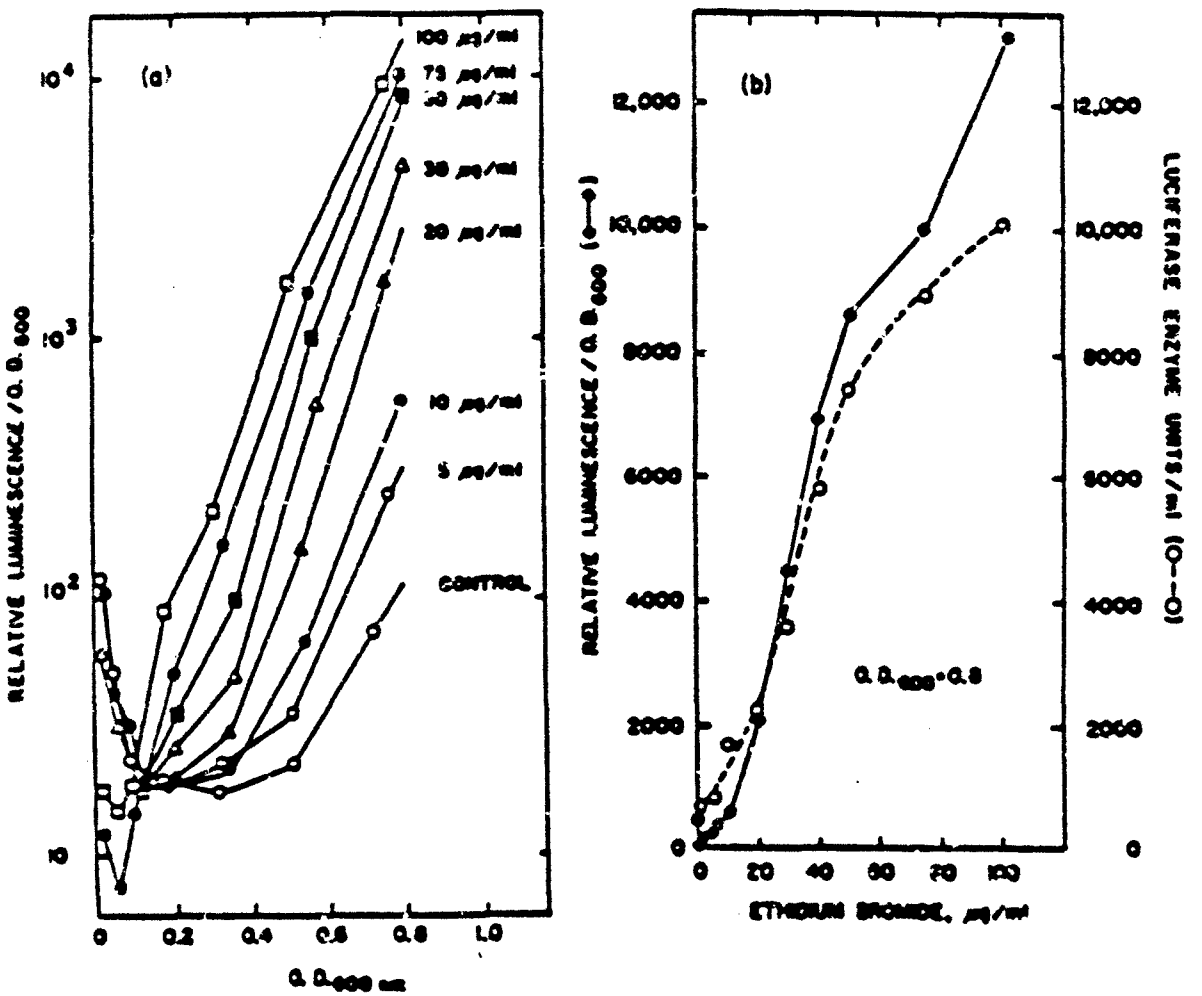

Figure 4. Response of B18(pSD721) cells to various levels of ethidium bromide. a) Effect of various levels of EtBr on light emission as a function of cell growth. b) a standard response curve of luminescence and luciferase to various levels of EtBr.

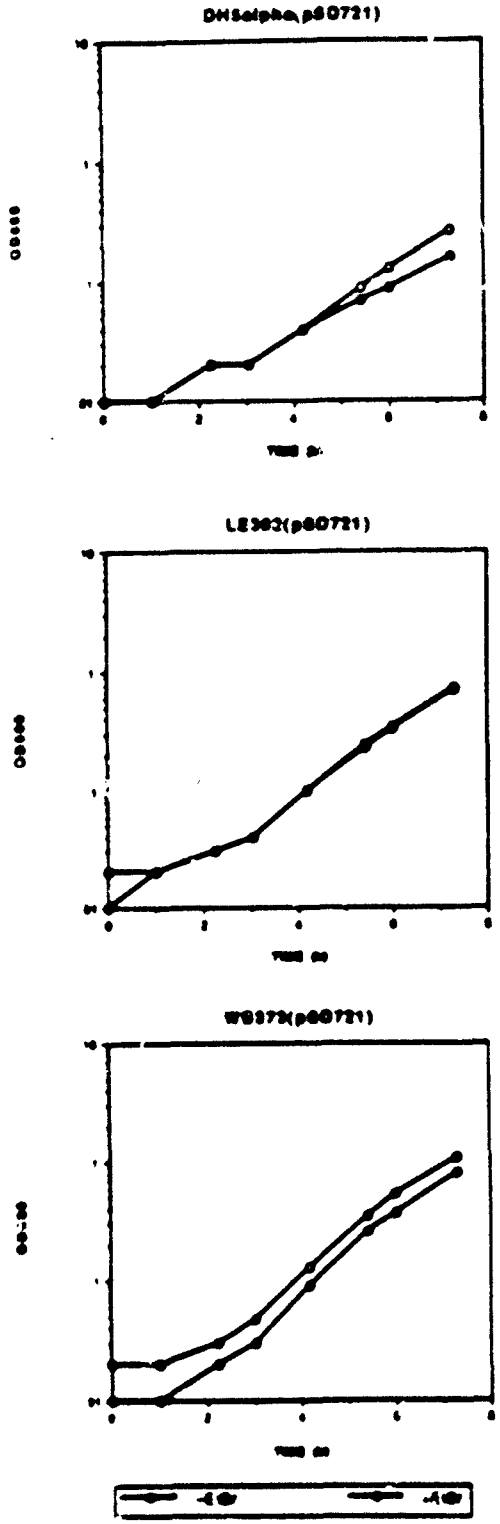

Figure 5. Growth of DH5α(pSD721), LE392(pSD721), and WB373(pSD721) with and witnout EtBr.

growth, in contrast to most enzymes. This "autoinduction" of luminescence illustrated when the luminescent response of a growing culture is plotted as luminescence per cell (luminescence/OD_{600}) vs. cell number (OD_{600})(Figure 6; see also Figure 3)(Nealson et al., 1970; Rosson and Nealson et al., 1981). All of the clones exhibited autoinduction of luminescence when grown with EtBr, but not without EtBr (Figure 6).

The growth studies verify that each of these clones is a potential carcinogen biosensor. In growth experiments, however, the luminescent response to carcinogens occurs over several population doublings and hence requires many hours to detect. Incubation also requires strict temperature control and vigorous agitation. These conditions limit the applicability of this type of test.

A rapid luminescent response to EtBr. Ideally, a luminescent biosensor will respond rapidly to the presence of a specific trace chemical and have a large luminous response relative to background luminescence. Exponential growth-phase cultures were sampled (OD_{600} = 1.0) for testing of the rapidity of response of these culture suspensions to 20 μg/ml EtBr (Figure 7). Generally, the luminous response to the addition of EtBr was measurable within 20 min, and maximal by 60 min (Figures 8-10). The affect of cell density on expression of luminescence was also determined. The luminescent response of these exponential growth-phase cultures was similar for suspensions of OD_{600} = 0.05 to 0.5 (Figures 8-10); use of low OD culture suspensions eliminated the requirement for agitation during the test for maximum expression of luminescence.

Luminescent response at different EtBr concentrations. Even at levels of EtBr as low as 2 μg/ml (2 ppm), the luminescent response was measurable (Figure 11). With DH5α(pSD721), a near maximal response, well above background luminescence, was observed within 45 minutes exposure to EtBr, for all concentrations of 2 μg/ml or greater. A similar luminescent response was noted for WB373(pSD721); the LE392(pSD721) response to 2 μg/ml EtBr was similar that of the no EtBr control. Using an appropriate clone, a highly sensitive response to carcinogens in the very low ppm range was easily achieved.

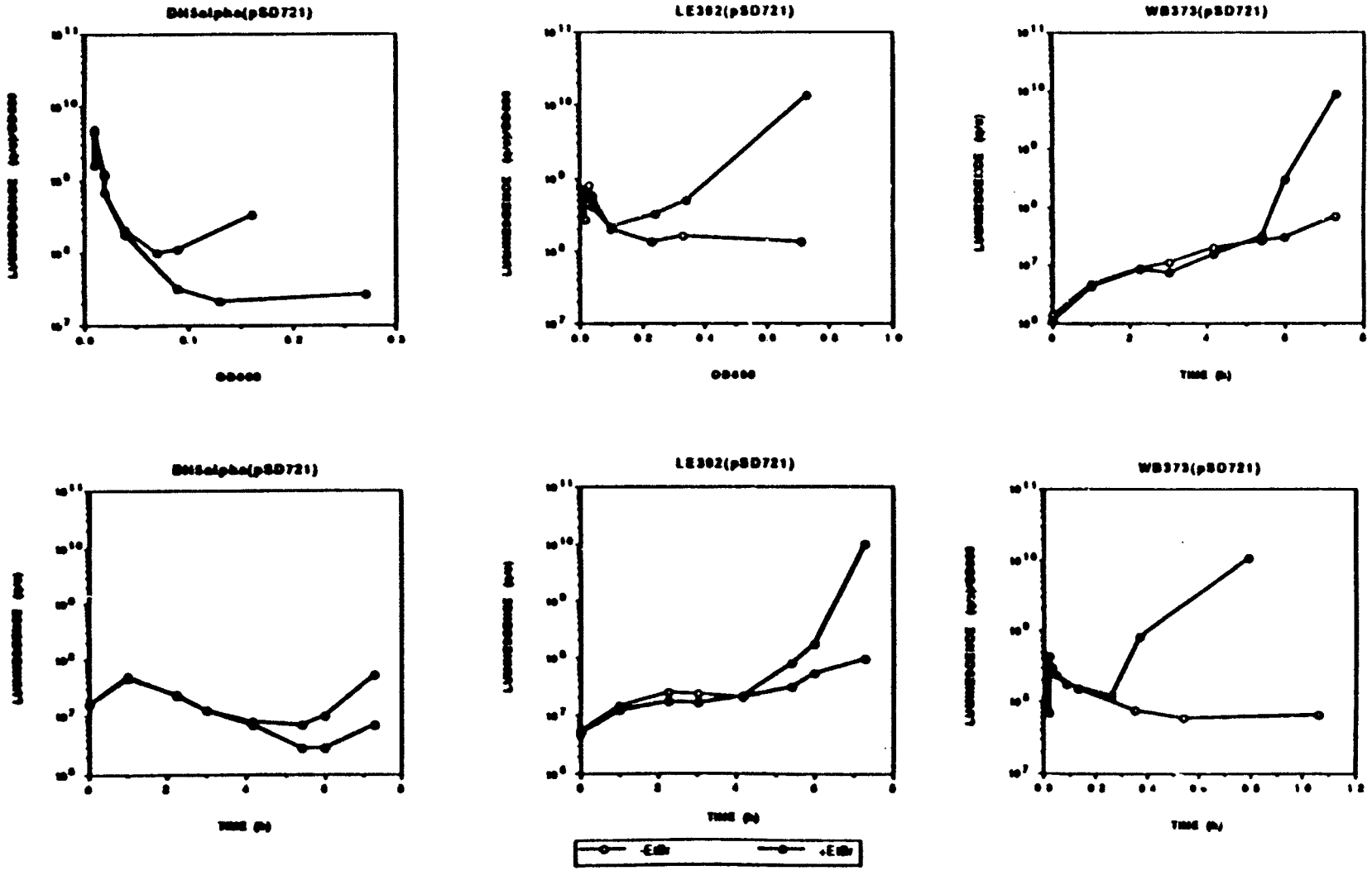

Figure 6. Autoinduction of luminescence for DH5α(pSD721), LE392(pSD721), and WB373(pSD721) with and without EtBr. The top panel shows luminescence per cell versus cell number. The bottom panel shows culture luminescence versus time.

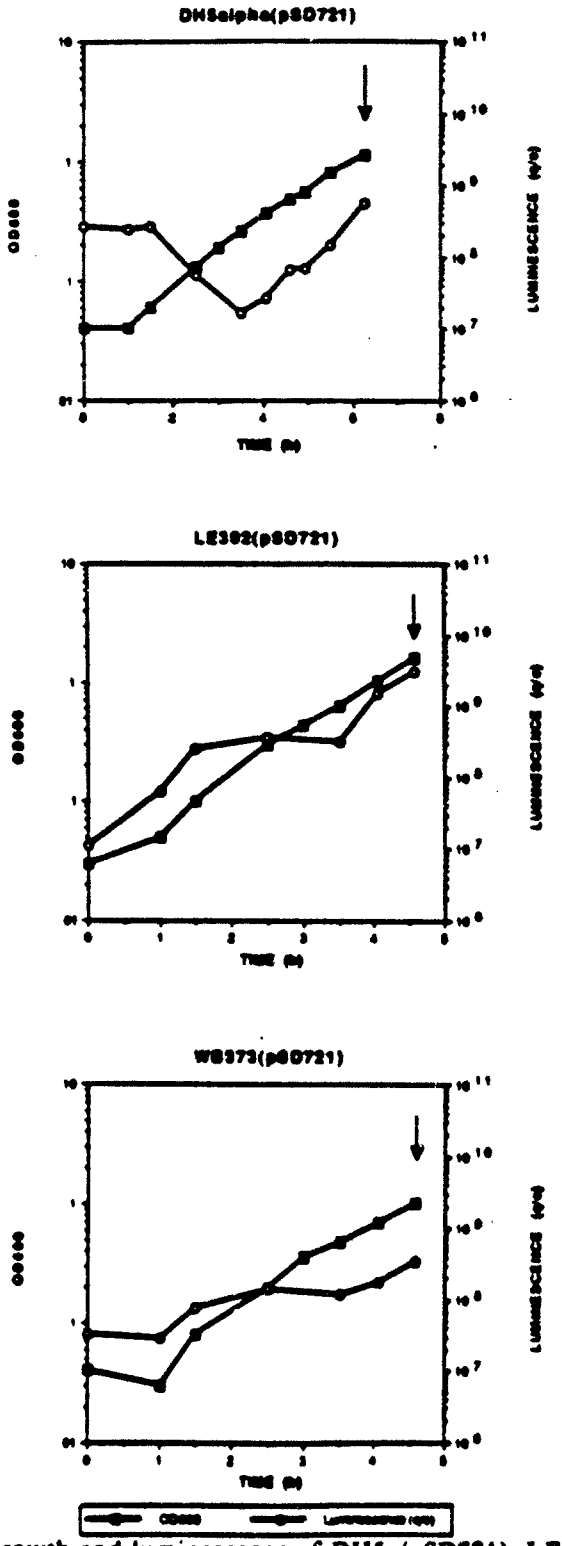

Figure 7. Growth and luminescence of DH5α(pSD721), LE392(pSD721), and WB373(pSD721) with and without EtBr as a function of time. The arrow shows the time of sampling for a rapid lumenescent response assay.

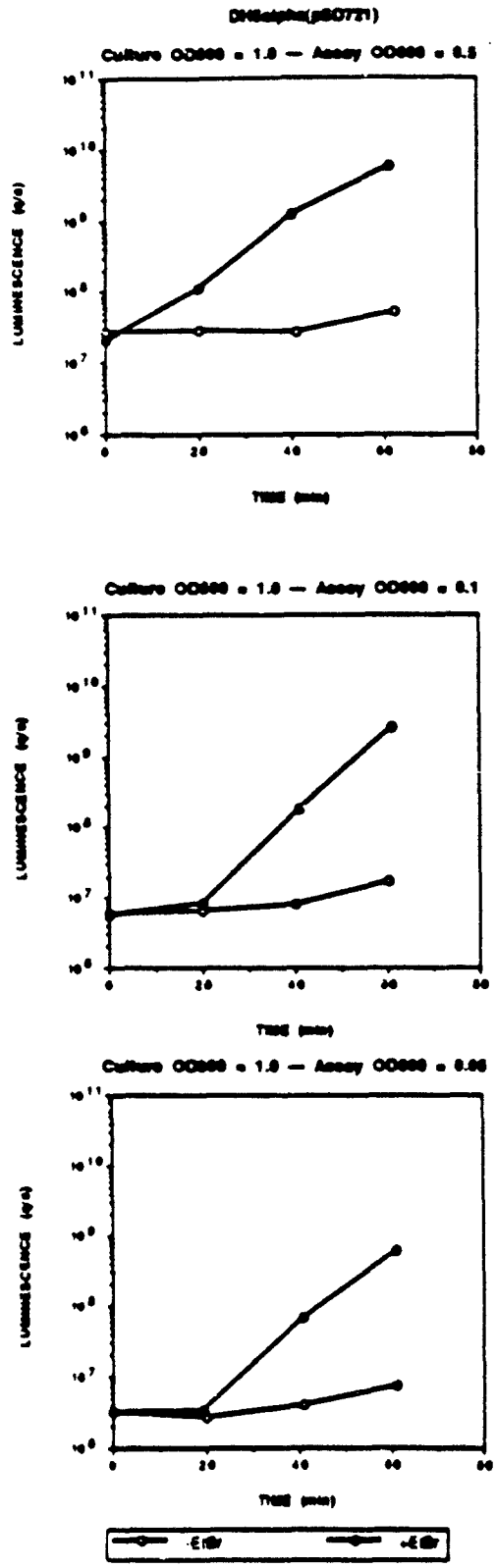

Figure 8. The rapid luminescence response of DH5α(pSD721) at different cell densities to EtBr.

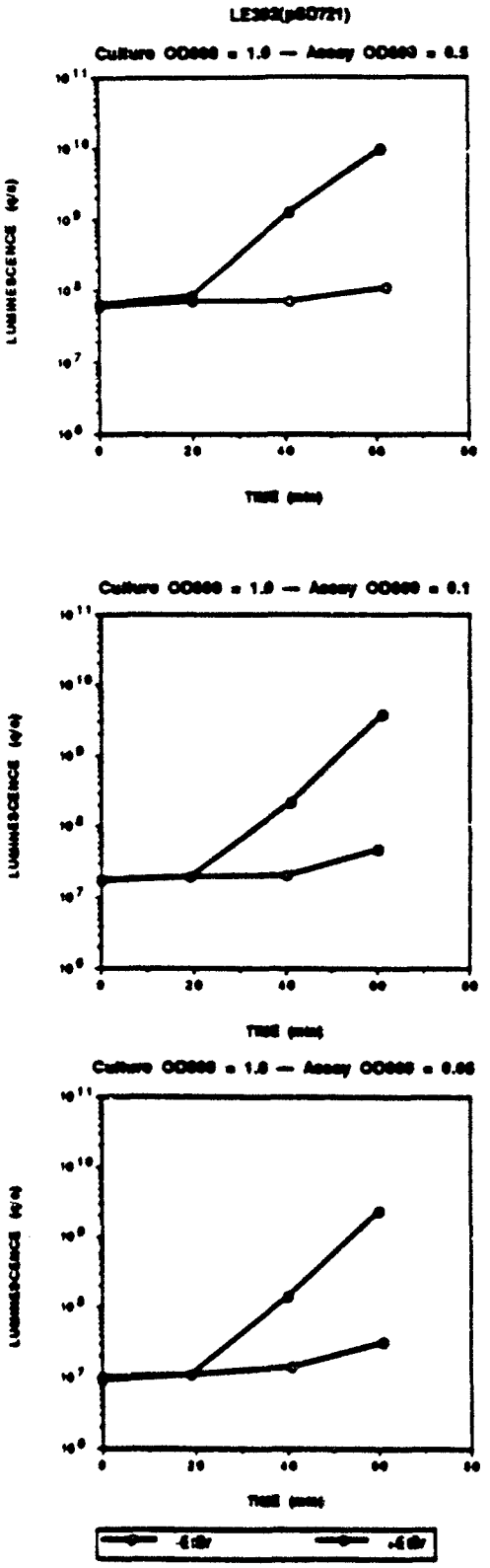

Figure 9. The rapid luminescence response of LE392(pSD721) at different cell densities to EtBr.

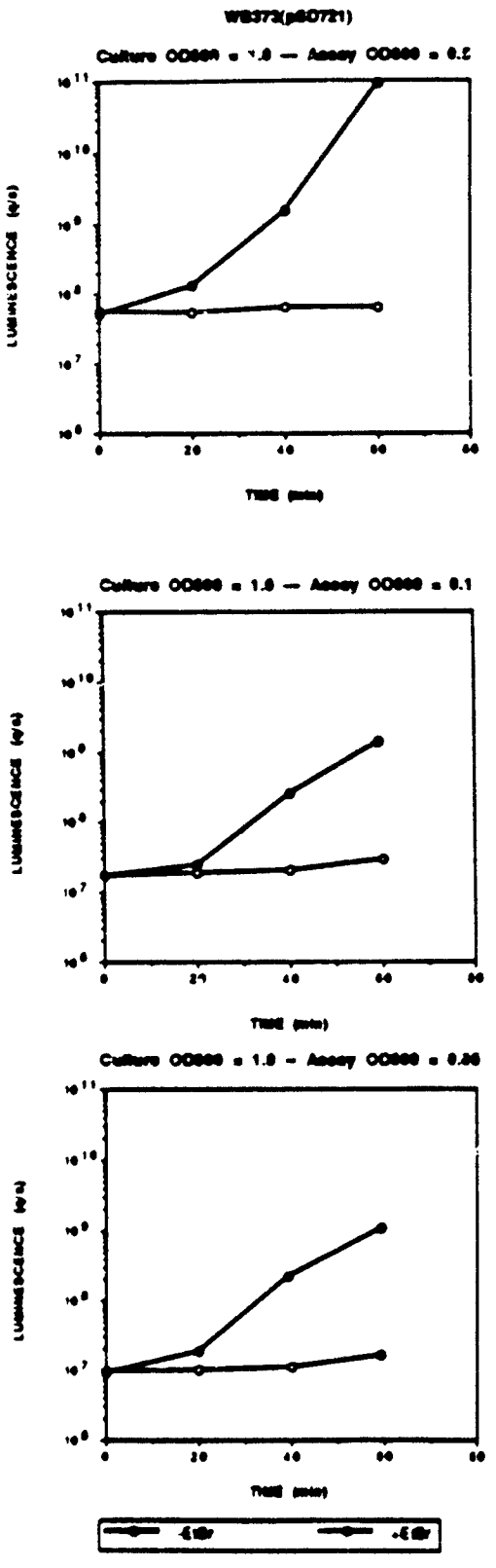

Figure 10. The rapid luminescence response of WB373(pSD721) at different cell densities to EtBr.

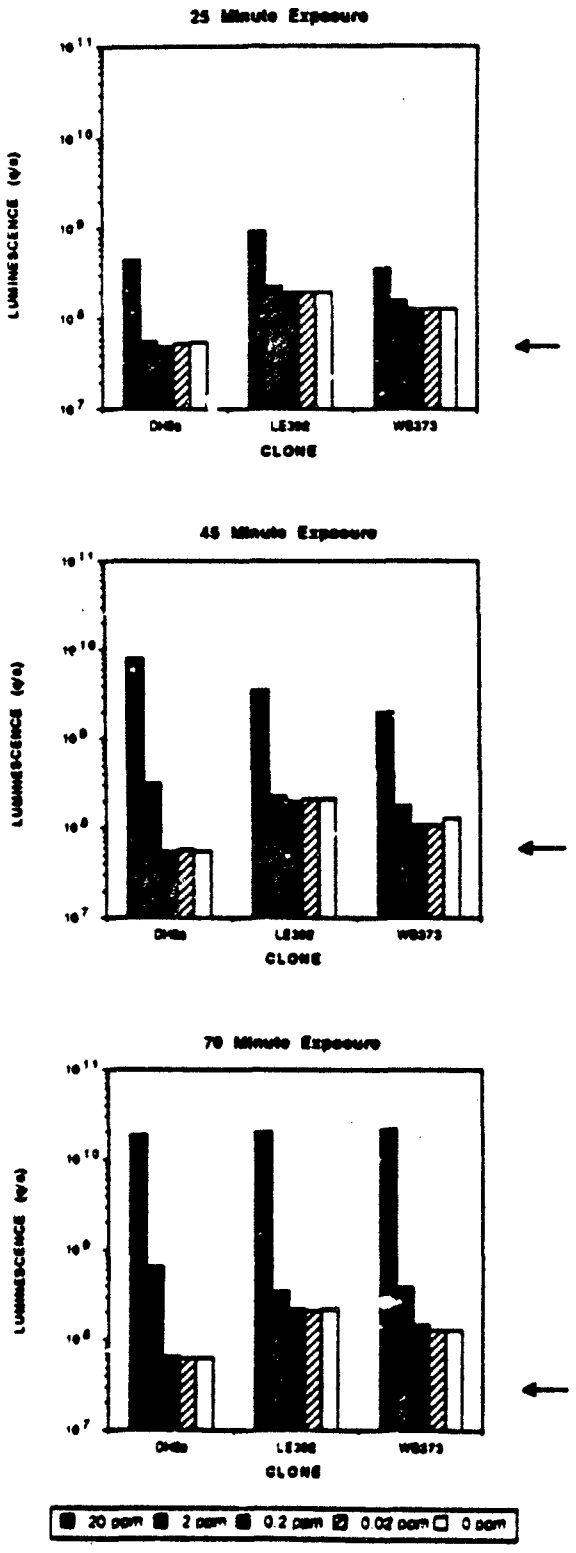

Figure 11. Luminescence response of DH5α(pSD721), LE392(pSD721), and WB373(pSD721) to concentrations of EtBr.

Selection of DH5α(pSD721) as a prototype carcinogen biosensor

A stable, rapid response to carcinogen, a low background luminescence, and a large luminescent response to carcinogen are all required for a working biosensor. The luminescent response of DH5α(pSD721) to EtBr was measurable over background within 10 minutes (Figure 8). The background luminescence of DH5α(pSD721) was 3 to 4 times lower than that of the other two clones (Figure 8). Generally, DH5α(pSD721)'s maximum luminescent response to EtBr was equal to that of the other two clones at most of the OD's tested. DH5α(pSD721) meets all of the requirements for a carcinogen biosensor. This genetically engineered clone is designated by BioPhotonics as Prototype Carcinogen Biosensor pSD721(027).

Luminescent response of DH5α(pSD721) at different stages of growth

To determine the growth conditions that yield the greatest potential for luminescent response to EtBr, DH5α(pSD721) was tested at different stages of growth. The luminescent response of DH5α(pSD721) to 20 μg/ml EtBr was measurable for suspensions taken at stages of growth spanning OD_{600} = 0.05 to 3.4 (Figure 12). Background luminescence increased with increasing culture OD. Dense suspensions (OD_{600} > 1) were oxygen limited and thus required vigorous agitation to achieve a stable luminescent response. Maximal luminescent response to EtBr, relative to the background, was observed for OD_{600} = 0.5 cultures. Luminescence was measurable within 5 minutes after the addition of EtBr. While exponential growth-phase cultures are all useful biosensors, it is clear that mid-exponential growth-phase cultures have the greatest potential to be extremely sensitive biosensors, as a rapid and a very large luminescent response occurs with the addition of carcinogens.

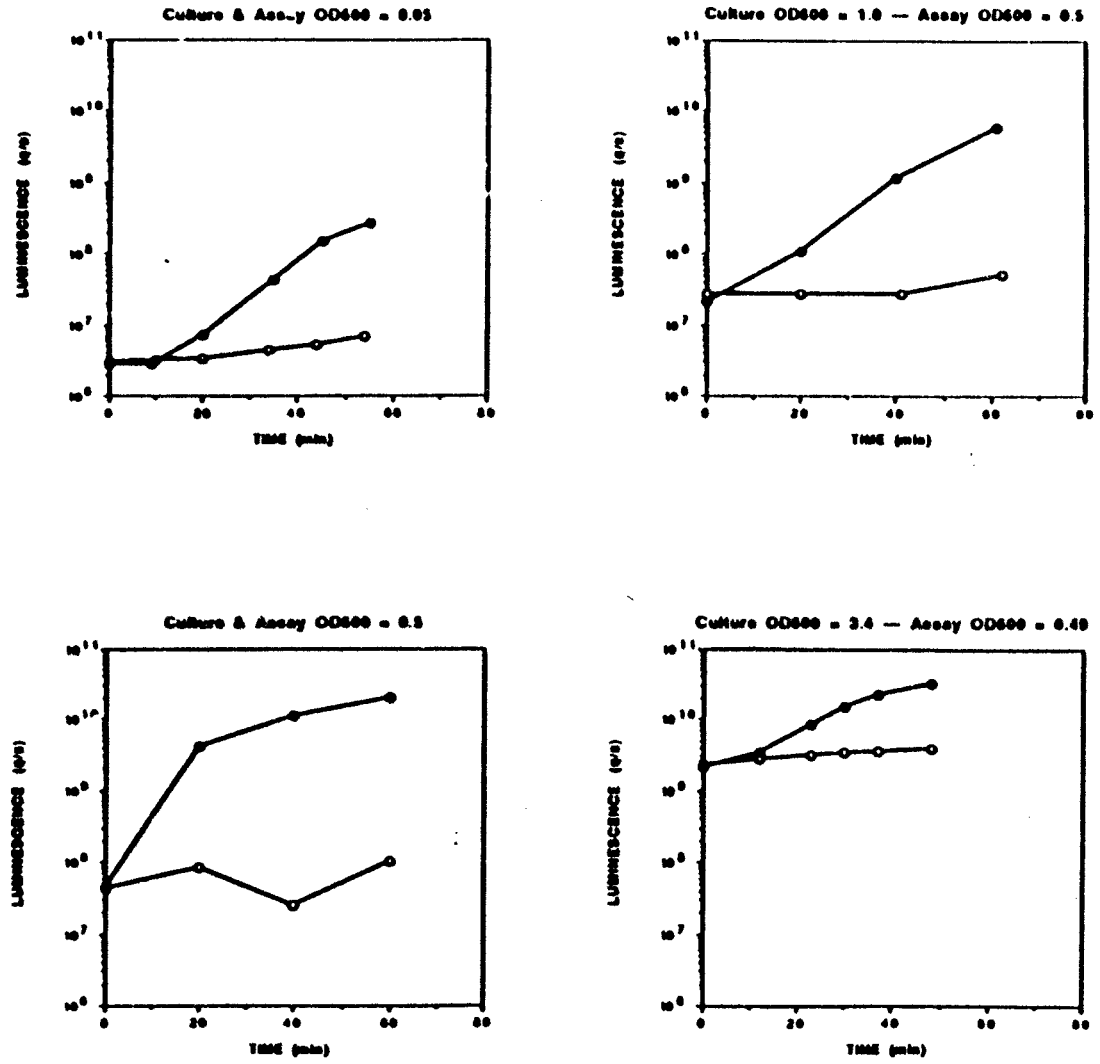

Figure 12. Luminescence response of DH5α(pSD721) to 20 μg/ml fo EtBr for cultures taken at different stages of growth.

Affect of conditioned medium diluent on the rapid luminous response

The growth media of some bioluminescent bacteria may contain factors that are either essential to, or stimulatory for, the expression of the luminescence genes (Eberhard et al., 1981; Nealson, 1977, Rosson and Nealson, 1981; Greenberg et al., 1979). Growth media cleared of bacteria by filtration through 0.2 μm membrane filters (conditioned media) have been shown, for some bioluminescent bacteria, to contain an autoinducer, and possibly the other factors necessary for expression of bioluminescence (Nealson, 1977; Greenberg et al., 1979). In anticipation that stabilized biosensor preparations will be stored as concentrates and thus need to be diluted upon rehydration, several conditioned media were tested for their suitability as diluents.

Conditioned medium, prepared from each of the clones grown to OD_{600} = 1.0, was used as diluent to monitor the response of each clone to EtBr (Table 2). The response of the DH5α(PSD721) clone was stimulated by use of both the LE392 and WB373 diluents. For the LE392(pSD721) clone, the maximum response was obtained using WB373 conditioned medium; while the WB373(pSD721) respo[illegible] maximum with the DH5α(pSD721) conditioned media.

It has been reported that a heat-labile factor is present in some conditioned media (Nealson, 1977; Greenberg et al., 1979). Experiments using boiled conditioned media showed untreated conditioned media to be equally effective.

Lyophilization

A freeze-drying technique was selected for initial stabilization and preservation of the cloned biosensors. All three clones have been successfully preserved and stabilized by lyophilization. Since DH5α(pSD721) was determined to be the most practical clone for carcinogen detection, extensive lyophilization studies were carried out using this culture. In preparation for lyophilization, DH5α(pSD721) was grown in LB at 30°C, to an OD_{600} = 1.0. The cultures were harvested and washed in 10 mM HEPES-buffered saline (pH 7.5). The cells were

Table 2. The effect of conditioned medium on the expression of luminescence by pSD721 clones in response to 20 μg/ml EtBr[a].

E. coli strain	Conditioned medium[b]	Luminescence without EtBr	Luminescence with EtBr	Stimulation (X)
DH5α	D	$1.5X10^7$	$2.1X10^8$	14
	L	$1.2X10^7$	$2.0X10^8$	100
	W	$1.5X10^7$	$1.2X10^9$	80
LE392	D	$4.7X10^7$	$1.9X10^9$	40
	L	$2.9X10^7$	$1.2X10^9$	41
	W	$7.0X10^7$	$7.6X10^9$	109
WB373	D	$2.0X10^7$	$1.9X10^8$	10
	L	$2.5X10^7$	$7.1X10^7$	3
	W	$3.0X10^7$	$9.3X10^8$	31

[a] pSD721 clones were grown to an OD_{600} = 1.0 and then diluted into conditioned medium to a final suspension OD_{600} = 0.1. A rapid-response test to 20 μg/ml of EtBr was performed at 30°C.

[b] Conditioned media consisted of OD_{600} = 1.0 cleared growth media from: D, (DH5α(pSD721)), L, (LE392(pSD721)), or W, (WB373(pSD721)). Media were cleared by centrifugation followed by filtration through sterile 0.2 μm-membrane filters.

then resuspended in 20% skim milk with either an appropriately conditioned media, the original growth medium, or a normal saline solution (0.85% NaCl), as noted in Table 3. These preparations were then frozen at -80°C and lyophilized using standard techniques. The lyophilized cells were stored at -80°C for as long as one and one-half months before being rehydrated.

Table 3. Response of DH5α(pSD721) clones to 20 μg/ml EtBr after lyophilization and rehydration.

Diluent for rapid response test	Lyophil-ization Diluent (see note)	Luminescence (q s^{-1})					
		OD_{600} = 0.05			OD_{600} = 0.5		
		without EtBr	with EtBr	Stimula-tion (X)	without EtBr	with EtBr	Stimula-tion (X)
20% skim milk	M	$1.7x10^7$	$7.4x10^7$	4	-	-	
	C	$7.4x10^6$	$6.5x10^6$	0	-	-	-
	S	$1.8x10^6$	$1.6x10^7$	0	-	-	-
Condi-tioned medium	M	$1.7x10^7$	$2.1x10^8$	19	$7.3x10^7$	$1.3x10^{10}$	178
	C	$1.0x10^7$	$1.2x10^8$	12	$8.3x10^7$	$5.8x10^9$	70
	S	$1.1x10^7$	$2.5x10^8$	23	$1.5x10^{10}$	$1.7x10^{10}$	0
Saline	M	$4.8x10^7$	$1.1x10^9$	23	$6.6x10^9$	$5.3x10^{10}$	8
	C	$1.7x10^7$	$1.3x10^9$	76	$4.3x10^8$	$7.7x10^{10}$	179
	S	$7.2x10^6$	$1.1x10^7$	2	$2.8x10^7$	$7.9x10^7$	3

NOTE: Lyophilization Diluents: M, (20% skim milk); C, (conditioned medium); and S, (saline).

Recovery of the luminescent response to EtBr after rehydration

The stored cells were rehydrated by addition of distilled water at 4°C, and then allowed to stand on ice for one hour. Following this, a small amount of the concentrated cells was placed in 20 ml vials and diluted to a volume of 1 ml at a final density of either OD_{600} = 0.05 or 0.5, by adding one of the three diluents listed in Table 3.

Upon rehydration of the stabilized cell material and dilution into the appropriate diluent, the luminescent response to EtBr was almost completely recovered (Table 3). The recovery of the luminescent response was best for those cultures that were suspended in saline plus skim milk for lyophilization, and when conditioned media were used as the diluent for the rehydrated clones;

the luminescent response was nearly identical to that of the live cells. Dependent on the suspension density, a 75 to 175 fold increase in the luminescence was obtained in response to the presence of EtBr, after 60 minutes.

The level of the luminescent response of rehydrated biosensors to EtBr after being lyophilized for 1.5 months was essentially identical to those samples rehydrated after only 24 hours of storage, and very nearly the same as for live cells. It should be noted that, because of the duration of the Phase I grant, the storage term that could be evaluated was severely limited to only 1.5 months. It is anticipated that the cells can easily be stored for several years without damage. Under Phase II funding the shelf-life, both frozen and at room temperature, will be more fully evaluated.

Mercury Biosensor Development

Cloned genes that respond to mercury (*mer* operon) were obtained as two M13 bacteriophage derivatives, mGN2-327 and mGN2-220 (Figure 13; Table 4). These phages contain part of the broad spectrum mercury resistance operon from a *Serratia* sp., originally in the plasmid pDU1358 (Nucifora et al., 1989). Cloned *lux* genes for mercury biosensor construction were from *Xhenorhabdis luminescence* (Figure 13).

The goal of this component of the Phase I research was to initiate the development of a biosensor specific for mercury, by fusion of the inducible regulatory promoter element of the *mer* operon to reporter *lux* genes. The component genetic elements to construct a mercury biosensor were obtained by BioPhotonics during Phase I, and work toward constructing the *mer/lux* fusion was begun. All cloning was by standard techniques (Maniatis et al., 1982).

Mer-containing M13 phage mGN2-327 and mGN2-220 were grown on *E. coli* WB373 overnight at 37°C as described by Nucifora et al. (1989). *Lux*-containing *E. coli* JM83(pCGLS11) was grown overnight at 30°C in LB plus chloramphenicol (30 μg/ml). Double-stranded phage RF DNA and plasmid pCGLS11 DNA were isolated by a rapid alkaline plasmid extraction (Krieg and Melton, 1984). The presence and orientation of *mer* genes in mGN2-327 and mGN2-220 and of *lux* genes in pCGLS11 was confirmed by restriction enzyme mapping. The *mer* genes were

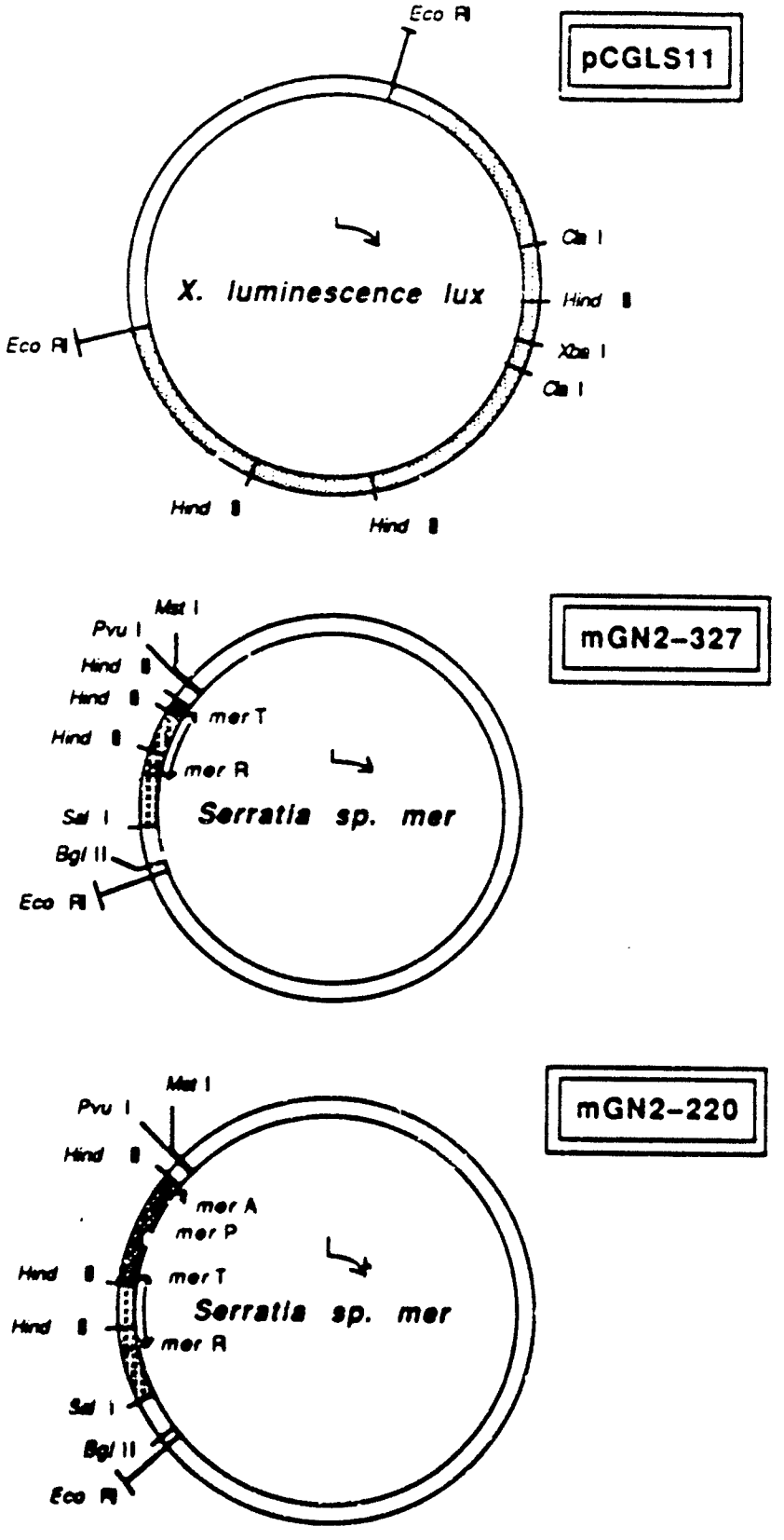

Figure 13. Map of the plasmid pCGLS11, mGN2-327, and mGN2-220.

Table 4. Strains, plasmids, and bacteriophage used in the mercury study.

Strain or Plasmid	Genotype	Source
E. coli Strain		
WB373	F^+, *tra*$^-$, *lac*$^+$, *Amp*^{R}	Silver
Plasmid/Phage		
pCGLS11	*luxC,D,A,B*; from *X. luminescens*	Frackman
mGN2-327	*merR, OP*, part of *T*; from pDU1358	Silver
mGN2-220	*merR, OP, T, P, A*; from pDU1358	Silver

excised and purified from the plasmids by electrophoresis in agarose.

Progress to date is very encouraging. The plan for completion of the cloning of *mer* into pCGLS11 includes: 1) linearization of the *lux* plasmid and 2) ligation of purified *mer* genes into the polylinker region of pCGLS11.

Light Detection Systems

The purpose of researching light detection systems was to develop a detector unit to conveniently measure the relative light outputs of the photoluminescent biosensor, as all currently available detectors are bulky, prohibitively expensive, or not field portable. BioPhotonics has to date developed three different types of detectors.

Photodiode Based Detectors

Several photodiode based light detection systems have been developed by BioPhotonics to measure the light output of biological materials. The most sensitive photodiode based (Hamamatsu 220-01) system incorporates a small silicon photodiode (2.4 X 2.4 mm -- active area)

and a carefully matched amplifier mounted in a protective, light-tight case to detect the output of the luminescent bacteria/water sample. A 2 ml borosilicate glass vial placed 1 mm above the surface of the detector is used to contain the sample and cloned biosensor. This detector is capable of sensing light output on the order of several nanowatts and can be successfully used to measure the output of fully induced cloned *lux* system response. The cost of this detector and accompanying 15 volt portable power supply is about $450. Other less sensitive, and hence much less expensive ($50) photodiode arrangements were tested and could be used if the output of the cloned biosensors is further optimized.

Photomultiplier Based Detectors

In order to garner a more accurate assessment of the relative light output and the success of the different optimization procedures, several portable PMT based systems are under development. One of these systems utilizes a Hamamatsu R636 photocathode which features a strong, extremely linear response to light in the 350 to 700 nm range. This GaAs(Cs) photocathode generates the same relative output level regardless of the peak wavelength of the luminescent response. A second system utilizing a Hamamatsu R1894 head-on multi-alkali photocathode is currently being evaluated since the relatively small detector, 10 mm, makes the system more attractive for field use. The dynodes, power supply, and detector can be incorporated into a small battery-powered package that would provide substantially more sensitive evaluation of biosensor response than is available using photodiode based systems. The component costs for these systems is about $1000, which makes them very attractive in laboratory/field testing applications where repeated use of film techniques would be more expensive.

Film Based Detectors

Film techniques, both Polaroid and 35 mm, are yet another means of recording the luminescent biosensor response. As shown in (Figure 14), the response of the cloned genes can be

qualitatively determined using a simple timed exposure and Polaroid film cartridge. The control contol sample when compared to the 60 minute response to EtBr (20 μg/ml) is barely visible. Dilution as high as 8 fold have an easily detectable respose in comparison with the control.

Individual tests could be very economically packaged using Polaroid film to allow for rapid evaluation of the test, meeting the needs of the consumer market as well as those of industrial users requiring only occasional tests. The feasibility of all three of these techniques has been proven under Phase I funding, and further development all of these is planned for Phase II.

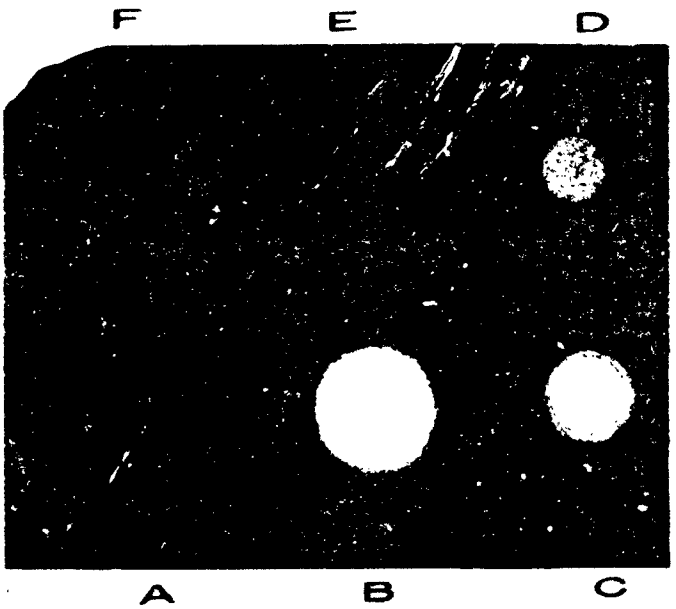

Figure 14. Time exposure on polaroid film of a) control luminescence, b) luminescent response to 20 μg/ml EtBr, c) a 50% dilution of b, d) a 75% dilution of b, and e) a 88% fold dilution of b.

CONCLUSIONS

The results of the Phase I investigation have met, and in many areas surpassed, the initial expectations of our proposal. These results include:

1) development of a near market-ready biosensor carcinogen detection system for use as a general screening test;

2) detection of Ethidium Bromide in the 1 μg/ml (1 ppm) range with a yet-to-be optimized genetically cloned sensor;

3) lyophilization and very successful rehydration of cloned biosensors after 1.5 months of storage;

4) early progress in the development of a mercury sensor, which establishes a methodology for the development of a variety of specific biosensors;

5) development of several light detection systems that include two photodiode based systems, two battery-powered photomultiplier system, and two film techniques.

SUMMARY

The techniques developed by BioPhotonics during Phase I can now be readily extended, with Phase II funding, to generate an array of luminescent biosensors for the detection of both broad classes of compounds and numerous specific trace chemicals. The carcinogen biosensor system will require only a single year of effort to optimize and characterize, to be ready for commercialization. BioPhotonics' initial progress toward construction of a specific biosensor for mercury, is very, encouraging; a *mer/lux* fusion based biosensor should be completed and ready for final development within a year. With Phase II funding, both sensors can be developed to a level ready for commercialization. Additionally, with Phase II funding and with the knowledge accumulated during Phase I, an array of other chemical specific biosensors can be constructed. BioPhotonics is especially interested in developing sensors for lead, chromium, phenol, benzene, and PCB's.

REFERENCES

Baldwin, T.O., and Shadel, G.S. (1988). Regulation of bioluminescence in Marine bacteria. Los Alamos: Diagnostics. 1:7-10.

Calvin, N.M., and Hanawalt P.C. (1988). High-efficiency transformation of bacterial cells by electroporation. J. Bacteriol. 170:2796-2801.

Delong, E.F., Steinhauer, D., Israel, A., and Nealson, K.H. (1987). Isolation of the *lux* genes from *Photobacterium leiognathi* and their expression in *Escherichia coli*, Gene. 54:203-210.

Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H., and Oppenheimer, N.J. (1981). Structural identification of autoinducer of *Photbacterium fischeri* luciferase, Biochemistry. 20:2444-2449.

Greenberg, E.P., Hastings, J.W., and Ulitzur, S. (1979). Induction of luciferase systhesis in *Beneckea harveyi* by other marine bacteria. Arch. of Microbiol. 120:87-91.

Hastings, J.W. and Weber, G. (1963). Total quantum flux of isotopic sources. J. Opt. Soc. Amer. 53:1410-1415.

Hastings, J.W. and Nealson, K.H. (1977). Bacterial bioluminescence. Annual Review of Microbiology. 31:549-595.

Hastings, J.W., Baldwin, T.O., and Nicoli, M.Z. (1978). Bacterial luciferase: assay, purification, and properties. *In* "Methods in Enzymology". (N.O. Kaplan and S.P. Colowick, eds.), Vol. 57, pp. 135-152, Academic Press, New York.

Krieg, P. and Melton, D. (1984). Nuc. Acids Res. 12:7057-7070.

Maniatis, T., Fritsch, E., and Sambrook J. (1982). "Molecular Cloning: A Laboratory Manual". Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Nealson, K.H. (1977), Autoinduction of bacterial luciferase: occurrence, mechanism, and signigicance. Arch. Microbiol. 112:73-79.

Nucifora, G., Chu, L., Silver, S., and Misra, T.K. (1989). Mercury operon regulation by the *merR* gene of the organomercurial resistance system of plasmid pDU11358. J. Bacteriol. 171:4241-4247.

Rosson, R.A. and Nealson, K.H. (1981). Autoinduction of bacterial bioluminescence in a carbon limited chemostat. Arch. Microbiol. 129:299-304.

Steinberg, D., Patterson, G.A., White, R.J., and Maiese, W.M. (1985). The stimulation of bioluminescence in *Photobacterium leiognathi* as a potential prescreen for antitumor agents. J. Antibiotics. 39:1401-1407.

Steinberg, D., Peterson, G.A., Nealson, K.H., and Maiese, W.M. (1988). Evaluation of bioluminescence as a prescreen for antitumor agents. "Advances in Chemiluminescence and Bioluminescence". Academic Press, N.Y.

Ulitzur, S. and Weiser, A. (1981). Acridine dyes and other DNA-intercalating agents induce the luminescence system of luminous bcteria and their dark variants. Proc. Nat. Acad. Sci. USA. 78: 3338-3341.

Ulitzur, S., Weiser, A., and Yanni, S. (1981). Bioluminescence test for mutagenic agents. "Bioluminescence and Cemiluminescence". Academic Press, N.Y.

Ulitzur, S. (1986). Bioluminescence test for genotoxic agents, Methods Enzym. 133:264-274.

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